

Award Number: DAMD17-96-2-6017

TITLE: A Novel Gene Gun-Mediated IL-12 Gene Therapy for Breast Cancer

PRINCIPAL INVESTIGATOR: Sun Ning Yang, Ph.D.

CONTRACTING ORGANIZATION: Powderject Vaccines, Incorporated
Madison, Wisconsin 53711

REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20000907 135

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1999	3. REPORT TYPE AND DATES COVERED Annual (15 Sep 98 - 14 Sep 99)	
4. TITLE AND SUBTITLE A Novel Gene Gun-Mediated IL-12 Gene Therapy for Breast Cancer (95 Cancer)			5. FUNDING NUMBERS DAMD17-96-2-6017	
6. AUTHOR(S) Sun Ning Yang, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Powderject Vaccines, Incorporated Madison, Wisconsin 53711 e-mail: nsyang@aol.com			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The overall goal of our research is to develop an immunological approach for breast cancer gene therapy. The results of the first year study, described in our previous Annual Report, show that gene gun-mediated IL-12 gene therapy is effective against breast tumors in mouse models. During the second year of this study we demonstrated that 4T1 tumor is weakly immunogenic, and it can induce a low level immune response. However, the anti-metastatic effect of IL-12 gene therapy against 4T1 tumor is not mediated by T cells, but rather involves NK cells. From several different immunomodulatory genes tested in combination with IL-12 gene therapy, IL-18 and ICE were found to be more effective in treatment of established TS/A breast tumor than IL-12 alone. In a separate, but strategically relevant approach for cancer gene immunotherapy, a striking antitumor effect was demonstrated in mice vaccinated with a gene encoding the tumor-associated antigen gp100 in combination with a GM-CSF gene. The results of this study strongly suggest that gene gun-mediated <i>in vivo</i> IL-12 gene therapy approach in combination with other immunological approaches may be developed as an effective and safe alternative to systemic IL-12 protein for treatment of breast cancer.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 34	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

☒ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

☒ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

☒ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

☒ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

☒ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

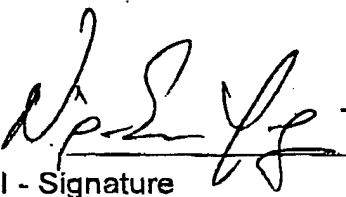
 Nov. 5, 1999
PI - Signature Date

TABLE OF CONTENTS

FRONT COVER	1
REPORT DOCUMENTATION PAGE	2
FOREWORD	3
TABLE OF CONTENTS	4
INTRODUCTION	5
BODY:	
Experimental Methods	6
Results	8
Discussion	13
KEY RESEARCH ACCOMPLISHMENTS.....	16
REPORTABLE OUTCOMES.....	16
CONCLUSIONS	17
REFERENCES	17
APPENDICES:	
Figure Legends	19
Tables	23
Figures	24

A. INTRODUCTION

Evaluated by many clinical investigators, cytokine therapy has been and is still actively considered as one of the more promising approaches for treatment of advanced forms of cancers, including breast cancer (1), because it can be directed at eradication of both the primary tumor and its metastases via activation of an antitumor immunity. Among various cytokines, IL-12 in particular exerted dramatic antitumor effects in several different experimental tumor models (2,3). Unfortunately, little information is available for mammary tumor models. In addition, recombinant IL-12 protein in therapeutic doses can be toxic to mice and humans (4,5). Directed by the Army Breast Cancer Research Program, we have focused our laboratory effort on the cytokine gene therapy for breast cancer.

The ultimate goal of our research is to develop an immunological approach for breast cancer gene therapy that can result in regression of both primary tumors and residual metastatic foci, and can also induce sufficient immunological memory to prevent tumor recurrence and progression. Based upon our previous gene gun studies, this strategy is expected to exploit the gene therapy potential for treatment of breast cancer with minimal or none toxic side effects encountered in other studies employing cytokine protein therapy (4,5).

Our laboratories previously reported (6,7) that gene gun-mediated *in vivo* delivery of IL-12 DNA elicited effective antitumor responses with no evident toxicity. This therapeutic effect was achieved via localized transgenic production of IL-12 protein, at a systemic level at least 1,000 times less than the effective, and toxic, dose of recombinant IL-12 protein delivered systemically (8). We therefore suggested that gene gun-mediated IL-12 gene therapy might be effective and non-toxic in murine mammary tumor models.

During the early stage of our gene therapy studies, it was shown in our previously published results that six out of six tested tumor models, including two sarcomas, a renal cell carcinoma, a lymphoma, a melanoma and a mastocytoma, responded at varying degrees to gene gun-mediated IL-12 gene therapy *in vivo* (6). Responses varied from complete regression to a significant suppression of tumor growth, depending on the immunogenicity of test tumors. These syngeneic mouse tumor models resemble two currently employed murine mammary tumor models, for which poorly immunogenic and highly immunogenic tumor cell lines have been characterized, and histology, tumorigenicity, and metastatic capacity recently established (9-13). Thus there was good reason to suggest that experimental murine mammary tumor systems may also be responsive to this gene therapy approach.

In essence, experimental results obtained from the past two and half years of this study have been described in our previous Annual Report. We showed that gene gun-mediated IL-12 gene therapy can be effective against breast tumors in mouse models. Briefly, these results are summarized as follows:

- 1) Gene-gun-mediated IL-12 gene therapy of the immunogenic TS/A adenocarcinoma can result in complete regression of some of the established primary tumors and induction of immunological memory.

- 2) This IL-12 gene therapy protocol for the non-immunogenic 4T1 adenocarcinoma does not significantly affect the growth of the primary tumor, but can reduce metastasis into the lungs.
- 3) The current IL-12 gene therapy can significantly extend the test mouse survival time following the excision of a 4T1 primary tumor.
- 4) The observed anti-metastatic effect of IL-12 gene therapy against the 4T1 tumor apparently did not involve T-cells, but appeared to involve NK cells and IFN- γ .
- 5) Co-delivery of a combination of IL-12, pro-IL-18 and IL-1 β converting enzyme (ICE) cDNA resulted in a synergistic, significantly enhanced inhibition of the growth of TS/A tumors.
- 6) The IL-12, pro-IL-18 and ICE combinational gene therapy resulted in high level of IFN- γ induction in vitro and in vivo, and was CD8+ cell dependent.
- 7) With a DNA vaccine approach, co-delivery of granulocyte macrophage colony-stimulating factor (GM-CSF) cDNA together with gp100 cDNA into skin reproducibly resulted in a much greater level of protection against challenge with a murine B16 melanoma stably transfected with human gp100 cDNA.

During the third year of this study we continued the characterization of the antitumor and anti-metastatic effects of IL-12 gene therapy against breast tumors. We focused on two main aspects: 1) develop and optimize new experimental protocols (e.g., combinational cytokine gene transfer) for augmenting the antitumor effect of IL-12 gene therapy, 2) develop DNA cancer vaccine approaches using human tumor associated antigen(s) and GM-CSF as DNA adjuvant. The results obtained to date have been quite encouraging, and are described as follows. Three scientific papers and one book chapter, including a paper in press in PNAS USA, have been generated so far from this study.

B. BODY OF REPORT

B1. Experimental Methods

cDNA Expression Plasmid Construction. The murine IL-12 expression plasmid, pNGVL3-mIL12, was constructed in a backbone plasmid containing a CMV immediate/early enhancer promoter, intron A and a kanamycin selection gene. The p35 and p40 subunits, separated by an internal ribosomal entry site, were subcloned into the multiple cloning site of pNGVL-3 (National Gene Vector Laboratory, University of Michigan, Ann Arbor, MI). The backbone plasmid, pNGVL-3, was used as a control vector. To construct a plasmid expressing pro-IL-18 or ICE cDNA, the full length of murine pro-IL-18 or ICE cDNA was obtained from total RNA of C57BL/6 mouse spleen cells by reverse transcription-polymerase chain reaction (RT-PCR) using a RT-PCR kit (Takara, Tsukuba, Japan). The PCR product of pro-IL-18 was subcloned into pNGVL-3 (pNGVL-3-mIL-18) and that of ICE was directly subcloned into the expression vector pCR3.1 (Invitrogen, Carlsbad, CA, USA) by TA cloning (pCR3.1-ICE). For B16 cell transfection, the construct pNASS/CMV-hgp100 was used. It was generated by inserting the hgp100 DNA fragment, excised from the pWRG1644 vector, into the pNASS/CMV-neo vector.

Mice and Cell lines. BALB/c mice and C57Bl/6 mice were obtained from Harlan-Spargue-Dawley (Madison, WI), or from Taconic (Germantown, NY) and were housed in an AAALAC-accredited facility under isothermal conditions and allowed access to food and water ad libitum.

Female mice between 8-12 weeks of age were employed. The cell lines were used in the studies included: TS/A (murine mammary adenocarcinoma), Lewis lung carcinoma (LLC), B16 (murine melanoma), RAW 264.7 (murine macrophage) and COS-7 cells. TS/A is a moderately immunogenic tumor and MHC class I positive (H-2Dd, H-2Kd). This cell line was originally obtained from Dr. G. Forni (Milan, Italy). The cell cultures were maintained in RPMI 1640 (Biowhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% minimal Eagle's medium nonessential amino acid, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Biowhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO₂ at 37°C. The B16 melanoma cell line, syngeneic in C57Bl/6 mice, was obtained from Dr. William Ershler (UW Institute of Aging, University of Wisconsin-Madison).

Murine Tumor Models and In Vivo Treatment Protocol. Mice were shaved in the abdominal area and injected intradermally with 5×10^4 - 1×10^5 tumor cells in 50 µl phosphate-buffered saline (PBS). We utilized a helium-pulse Dermal PowderJect-XR (formally Accell®) gene gun (PowderJect Vaccines, Inc., Madison, WI) as previously described (6). Briefly, plasmid DNA was precipitated onto gold particles 2 µm in size (Degussa, South Plainfield, NJ) at a loading rate of 2.5 µg DNA/mg of gold and coated onto the inner surface of Tefzel tubing. Each half-inch segment of tubing conferred the delivery of 0.5 mg gold and 1.25 µg total plasmid DNA per transfection. When two or three plasmids were used in combination, each plasmid was precipitated onto separate gold particles, which were then mixed together. In some experiments, hgp100 DNA was diluted 10-fold or 100-fold before precipitating onto gold particles. For DNA vaccination or treatment, mouse skin was shaved and transfected *in vivo* with the cDNA expression vectors with a 400 pounds per square inch (psi) helium gas pulse. For TS/A tumor treatment, mouse skin overlying and surrounding the tumor was transfected in with cytokine cDNA expression vectors and control plasmid (pNGVL-3) on days 6, 8, 10 and 12 after tumor implantation. There were six groups of gene gun treatments, which included: (I) mIL-12 cDNA alone; (II) murine pro-IL-18 cDNA alone; (III) murine pro-IL-18 and ICE cDNA (pro-IL-18 / ICE); (IV) mIL-12 and pro-IL-18 cDNA (IL-12 / pro-IL-18); (V) mIL-12, pro-IL-18 and ICE cDNA (IL-12 / pro-IL-18 / ICE); and (VI) control vector (pNGVL-3). Each treatment consisted of four transfections. Tumor growth was monitored two to three times per week by caliper measurement of two perpendicular tumor diameters. Mice were sacrificed when the diameter of the tumor reached 10 mm or greater. Mice that had been tumor free for more than 50 days after tumor implantation were challenged with an intradermal injection of 1×10^5 of the parental tumor cells (TS/A) or CT26 tumor cells (murine colon adenocarcinoma), which is also syngeneic to BALB/c mice.

In Vitro Gene Transfer and IL-18 Bioassay. For *in vitro* transfection, 2.5×10^6 of COS-7 or TS/A cells were suspended in 30 µl RPMI-1640 and spread into the target size in a 35 mm dish. Transfections with cytokine cDNA were performed using a gene gun with 250 psi helium pulse. Cells were then recounted using the trypan blue dye exclusion assay and 1×10^6 viable cells were placed in 2.0 ml of culture medium. Culture supernatants were harvested at 24 hr post-transfection for use in the IL-18 bioassay. The relative bioactivity of IL-18 was determined by the ability of cell supernatants to augment IFN-γ production *in vitro* (14). Briefly, mouse splenocytes (2×10^6) were cocultured with Con A (1.25 µg/ml) in 24-well plates for 48 hr. Supernatants obtained from transfected cells were added to cell suspensions of Con A-primed splenocytes (1×10^6) in 96-well plates for 24 hr. The supernatants were collected and assayed by ELISA to detect IFN-γ production.

In Vivo Depletion of CD4⁺ and CD8⁺ T cells and Neutralization of IFN- γ

The relative contribution of T cell subsets and IFN- γ was evaluated by in vivo antibody inhibition. Anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) were administered intraperitoneally at 0.3 mg per injection per mouse on days 5, 9 and 13 after tumor implantation (6). Anti-IFN- γ mAb (clone R4-6A2) was injected intraperitoneally at 0.5 mg per mouse on days 5, 7, 9, 11 and 13. As a control antibody, rat IgG (Sigma) was injected at 0.5 mg per mouse with the same schedule as for the anti-IFN- γ mAb.

Cytotoxic Assay. Spleen cells (8×10^6), derived from BALB/c mice that rejected TS/A tumors following gene therapy and remained tumor-free for at least 50 days, or obtained from TS/A tumor bearing mice 2 weeks after tumor implantation, were co-cultured with irradiated TS/A cells (4×10^5) for 5 days *in vitro*. TS/A cells labeled with $\text{Na}_2(^{51}\text{Cr})\text{O}_4$ (5×10^3 / well) were cultured in a total volume of 200 μ l with effector cells in 96 round-bottomed well plate. After 4 hours of incubation, the supernatant was harvested and counted in a gamma counter, and specific lysis was calculated (6).

PCR. C57/BL6 mice were vaccinated using the gene gun method as described below. One transfection contained a total of 625 ng of DNA of either vector alone, or vector encoding hgp100, or vector encoding hgp100 mixed at a 1:1 ratio with vector encoding mouse GM-SCF. Twenty four hours later, skin samples were taken from the transfected area, and total RNA was extracted using the guanidinium method.

The RNA was quantified spectrophotometrically and tested for DNA contaminations by running a PCR using 40ng of RNA as template and the primers listed below. All RNA was tested for DNA contamination, and RNA that showed signs of DNA contamination was subjected to DNase treatment according to standard protocols. Single stranded cDNA was generated from RNA with oligo-dT priming and AMV-RT according to the manufacturers instructions (Promega, Corp., Madison, WI). The resulting cDNA was amplified using primers obtained from Integrated DNA Technologies Inc., Coralville, IA. One set of primers was designed to specifically amplify human gp100 (upper 5'-TATTGAAAGTGCCGAGATCC-3'; lower GCTGTTCTCACCAATGGGACAAG-3'), the other primer amplifies both human and mouse gp100 (upper 5'-TATTGAAAGTGCCGAGATCC-3', lower 5'-TGCAAGGACCACAGCCATC-3'). The conditions for the PCR reaction were: initial denaturation step 4 min at 94°C; 35 cycles of denaturation (94°C for 45 sec), annealing (55°C for 45 sec) and extension (72°C for 1.5 min), and a final extension at 72°C for 7 min. The products were evaluated by 1.4% agarose gel electrophoresis in ethidium bromide and visualized by UV light.

Flow cytometry. The parental (B16-wild) and hgp100-transfected B16 cells (B16-gp100) were first permeablized in ORTHO PermaFix™ solution (Ortho Diagnostics Systems, Raritan, NJ) for 1 hr, followed by incubating with anti-gp100 mAb, HMB-45 (Adema et al., 1993), obtained from Coulter-Immunotech (Westbrook, Me), for 40 min at 4°C. As negative control, MOPC IgG (Sigma, St. Louis, MO) was used. After washing, the cells were stained with FITC-conjugated goat-anti-mouse IgG (Becton Dickinson and Co., San Jose, CA) for 40 min at 4°C. Stained cells were analyzed using a FACScan cytofluorometer (Becton Dickinson and Co., San Jose, CA) and data collected for 10,000 events/sample.

B2. Results

I. Combinational (IL-12, pro-IL-18, ICE) Gene Therapy Approach.

I.1. Induction of IFN- γ by gene transfer of IL-12, pro-IL-18 and/or ICE.

We hypothesized that expression of endogenous ICE in tumor cells may result in cleavage of the transgenic pro-IL-18. To evaluate this hypothesis, we transfected COS-7 cells (ICE negative) and TS/A (ICE positive) cells with pro-IL-18 cDNA alone or in combination with an ICE cDNA expression vectors. The ability of these cells to stimulate IFN- γ production in Con A-primed murine splenocytes was utilized as a bioassay to measure the relative levels of functional IL-18 protein (14-16). Fig. 1 shows that IFN- γ level induced by transfection with pro-IL-18 and ICE was considerably higher than that by pro-IL-18 alone in both COS-7 and TS/A cells. As expected, the cotransfection of ICE and pro-IL-18 cDNA was superior to pro-IL-18 cDNA alone and resulted in enhanced bioactivity of IL-18 in the ICE negative cell line COS-7 (15). Both of the low level of endogenous ICE in TS/A cells and subsequent low level of IFN- γ production, however, were significantly enhanced by ICE cDNA cotransfection. Thus, dual transfection of pro-IL-18 and ICE cDNA results in secretion of more bioactive IL-18 protein than that of pro-IL-18 cDNA alone. The combination of IL-12, pro-IL-18 and ICE resulted in the highest level of IFN- γ release from Con A-primed splenocytes among all groups in COS-7 and TS/A cells (Fig. 1). In contrast, the IFN- γ level induced by pro-IL-18 and IL-12 together was not statistically different from that of treatment with IL-12 alone. Taken together, in terms of IFN- γ induction, IL-12 was synergistic with the IL-18 produced from ICE-cleaved pro-IL-18, but not with pro-IL-18 alone. Similar results were obtained in transfection studies performed with LLC and B16 cells (data not shown). To investigate whether IFN- γ levels could be augmented *in vivo* following the transfer of IL-12, pro-IL-18 and ICE cDNA, IFN- γ production in the gene gun-treated skin tissues and serum of test mice and in conditioned medium of cultured splenocytes were compared among various treatment groups. The IFN- γ level produced from the skin in the group of IL-12 / pro-IL-18 / ICE was the highest among all tested groups (Fig. 2A). When splenocytes freshly isolated from test mice were cultured for 24 hours in the absence of Con A, the level of IFN- γ secretion was greatest in the group that was treated with the set of IL-12, pro-IL-18 and ICE cDNA in combination (781 pg / 10^6 splenocytes / 24 hours, Fig. 2B). This result suggests that splenocytes can be significantly activated following *in vivo* gene delivery of IL-12, pro-IL-18 and ICE, and that gene gun-mediated skin transfection with IL-12, pro-IL-18 and ICE cDNA may be capable of stimulating immune cells systemically as well as locally.

I.2. Antitumor effect of gene gun treatment with IL-12, pro-IL-18 and ICE cDNA.

The highest level of IFN- γ induction via the combination of IL-12, IL-18 and ICE treatment among all tested sets, *in vitro* and *in vivo*, led us to test whether this combination could also result in regression of established TS/A tumors. The combination gene therapy of IL-12, pro-IL-18 and ICE elicited the most marked suppression of tumor growth among all groups (Fig. 3). Even more important, a complete regression was observed on day 50 in 11 out of 22 tested mice (50.0%) in the IL-12 / pro-IL-18 / ICE combinatorial gene therapy group, and this rate was 2- to 3-fold higher than that obtained from treatment with IL-12 alone, or IL-12 / pro-IL-18 (Table 1). These results further confirm that the combination of IL-12 / pro-IL-18 / ICE in this gene therapy strategy can confer a much more efficacious antitumor activity than that from either the IL-12 gene alone or IL-12 / pro-IL-18 genes. This is the first evidence that a combinatorial direct treatment with IL-12 and IL-18 cDNA can result in complete eradication of established and relatively large tumors.

I.3. Involvement of CD8 $^{+}$ T cells and IFN- γ in Tumor Regression Following the

Combinatorial Gene Therapy of IL-12, pro-IL-18 and ICE. To determine the mechanism of cytokine-mediated tumor regression, *in vivo* depletion experiments were performed by injecting TS/A tumor-bearing mice with anti-CD4 $^{+}$, anti-CD8 $^{+}$ or anti-IFN- γ mAb (Fig. 4). Mice treated with IL-12 / pro-IL-18 / ICE and both anti-CD4 $^{+}$ and CD8 $^{+}$ mAbs were found to develop tumors

with kinetics similar to that of the control plasmid group (Fig. 4A). In vivo depletion of CD8+ T cells, but not of CD4+ T cells, completely abrogated the antitumor effect of combination therapy (Fig. 4B). These results suggest that tumor regression induced by this combinatorial therapy requires CD8+ T cells.

In vivo neutralization of IFN- γ proteins with anti-IFN- γ mAb also inhibited the antitumor activity against the TS/A tumor induced by IL-12 / pro-IL-18 / ICE cDNA, although this inhibitory effect was not complete (Fig. 4A).

I.4. CTL Activity Induced by Gene Gun Delivery of Cytokine cDNAs. To further evaluate whether the mice that showed complete regression of tumor following in vivo gene transfer were able to develop antitumor immunity, splenocytes collected from mice with complete regression in each treatment group were assayed for CTL activity. Splenocytes from IL-12 alone, IL-12 / pro-IL-18, and IL-12 / pro-IL-18 / ICE gene-treated mice generated similar levels of CTL activity, which were 4- to 6-fold higher than those from control tumor-bearing mice ($p < 0.01$, Fig. 5).

I.5. Immunological Memory in Mice Following Gene Therapy of IL-12, pro-IL-18 and ICE. We evaluated whether the mice that showed complete regression of TS/A tumors following gene therapy of IL-12 / pro-IL-18 / ICE could develop tumor-specific immunity. Six Balb/c mice with complete regression of TS/A tumor following in vivo gene gun treatment with IL-12 / pro-IL-18 / ICE cDNA were rechallenged on day 50 with 1×10^5 TS/A cells. All mice rejected TS/A tumors and were tumor-free for an additional one month. Then, these mice were rechallenged again with 1×10^5 of both TS/A cells and CT26 cells (syngeneic to BALB/c mice) on the right and the left side of abdomen, respectively. All mice rejected a second challenge with TS/A tumor cells but developed CT26 tumors (data not shown). Taken together with the data of the cytotoxic assay (Fig. 5), our results suggest that gene gun treatment with combination of IL-12, pro-IL-18 and ICE in tumor-bearing mice can effectively result in the CTL activation and the development of tumor-specific immunological memory.

II. Gp100+GM-CSF Gene Vaccination Approach

II.1. hgp100 expression *in vitro* and *in vivo* in B16 tumors and in the skin following particle-mediated hgp100 DNA transfection. Before evaluating antitumor efficacy of gp100 DNA vaccination, it was necessary to determine whether cell transfection with the hgp100 cDNA expression plasmid results in production of transgenic hgp100 protein. B16 cells were transfected with hgp100 cDNA, selected in vitro for positive clones (B16-gp100), and then analyzed for hgp100 expression using flow cytometry. Wild type B16 cells (B16-wild) and B16 cells transfected with empty vector (B16-neo) were used as controls. Because gp100 is a transmembrane glycoprotein, cell membrane was permeabilized prior to staining with hgp100-specific HMB-45 mAb. The results in Figure 6A show that B16-gp100 cells expressed more gp100 than control mouse B16 melanoma cells, indicating that the B16-gp100 clone expresses hgp100. The fact that nontransfected mouse B16 cells were also stained with HMB-45 mAb strongly suggests that this mAb recognizes an epitope shared between human and mouse gp100, since these proteins have approximately 80% homology (17). A non-melanoma murine cell line, 4T1 adenocarcinoma, was not stained with HMB-45 mAb (data not shown).

In a second approach, we looked at the expression of hgp100 in B16-gp100 cells by using RT-PCR analysis. Several previously published primer sets for the human gp100 sequence proved unsuitable in our system because of their cross-reaction with the mouse gp100, which has a 79.7% homology with the human sequence (17). We therefore designed a new set of primers that is able

to amplify only the human gp100 from hgp100-transfected, mouse gp100-positive cells. RT-PCR tests using the human-specific primer set confirmed its specificity, in that only the human gp100 transfected cell line, B16-gp100, resulted in an amplification product (Fig 6B, Lane 2) but not B16-wild (Fig. 1B, Lane 1).

In order to confirm the transcription of DNA that is delivered via gene gun into the skin of mice, C57/BL6 mice were treated with empty vector as a control, or with hgp100 DNA-coated gold beads, or with a mixture of hgp100 and GM-CSF-coated beads. The mice were given one transfection on each side of the abdomen, using 625 ng (hgp100) or 1250 ng (hgp100+GM-CSF) of total DNA per transfection. Twenty four hours later, skin samples were removed from the transfected area and total RNA was extracted. The RNA was tested for DNA contamination and then reverse transcribed and amplified by PCR using primers for the house keeping gene G3PDH and the hgp100. Lane 3 in Fig.6B shows that no hgp100 was detected in the empty vector-transfected skin, indicating that the specificity of the primers for the hgp100 sequence is high and no mouse derived products are interfering with the human-specific primers. A clear band in Lane 4 shows the positive amplification product of hgp100-transfected skin. Lane 5 confirms the expression of hgp100 when transfected along with GM-CSF, indicating that GM-CSF does not interfere with the expression of hgp100 in the skin. The integrity of all the cDNA samples was tested and confirmed by amplifying the house keeping gene G3PDH (Panel b). Thus, these data show that hgp100 DNA vaccination results in transgene transcription in the skin tissues 24 hr post-transfection.

II.2. Protection against B16-gp100 tumor following gp100 gene vaccination

The skin of mice was transfected at four abdominal sites with hgp100-encoding plasmid DNA, alone or in combination with mGM-CSF-encoding plasmid DNA. This resulted in a total delivery of 2.5 μ g of each DNA. Seven days later, vaccinated or naive mice were challenged intradermally (i.d.) in the middle of the abdomen with 5×10^4 of either B16-wild cells, or B16-gp100 cells, and tumor growth was followed. No significant tumor protection was observed against wild-type B16 tumor (Fig.7, left panel), or B16-neo tumor (data not shown) following either gp100 or gp100+GM-CSF gene vaccination. In contrast, gp100 gene vaccination resulted in a substantial protection against B16-gp100 tumors, with 40% of mice remaining tumor-free for at least two months. Importantly, co-delivery of GM-CSF cDNA with hgp100 cDNA resulted in a complete tumor protection in all 5 vaccinated mice (Fig. 7, right panel). This ability of GM-CSF gene co-delivery to enhance the effect of gp100 DNA vaccination was consistently reproducible in all twelve subsequent experiments, although the degree of protection varied. Marginal but statistically significant suppression of tumor growth could be achieved against B16-wild tumor in some experiments, especially when mice received two additional boosts following vaccination with the GM-CSF-gp100 gene combination (data not shown).

II.3. Dose effect of gp100 cDNA gene vaccination.

Because of the observed enhancing effect of GM-CSF on gp100 gene vaccination, we determined next whether the reduction of gp100 cDNA dose in a combined DNA vaccine would still result in tumor protection. The vaccination was designed as follows: the initial gp100 cDNA dose (625 ng per transfection) was decreased 10-fold and 100-fold, while GM-CSF cDNA dose remained constant (625 ng per transfection). Control mice received a vaccination with the empty vector alone and in combination with GM-CSF DNA. Seven days post vaccination, mice were challenged with 5×10^4 B16-gp100 cells, and tumor growth was followed. The results in Fig.8 show that, in combination with GM-CSF gene, a 10-fold reduced dose of gp100 plasmid DNA (62.5 ng) still induced an appreciable level of protection, which was less than that induced by 625 ng of gp100 DNA+ GM-CSF, but still much higher than gp100 DNA alone. Fig.8 also shows that

the protection against B16-gp100 tumors, although substantial, was not strong enough to completely eradicate the tumor in all vaccinated mice, in that some mice eventually developed the tumors several weeks after the tumor challenge.

II.4. Role of T cells in tumor protection in gp100 + GM-CSF DNA -vaccinated mice.

We investigated next whether T cells were responsible for the protection induced by gp100-GM-CSF gene vaccination. Mice were transfected in the skin with the GM-CSF-gp100 combined DNA vaccine on day 0, and injected i.p. with a mixture of anti-CD4 and anti-CD8 mAbs one day before and 5 days after B16-gp100 tumor challenge on day 7 post vaccination. Control mice received rat IgG. The results in Figure 9 show that *in vivo* depletion of T cells abrogated the protection induced by the DNA vaccine, indicating that this protection is T cell mediated. In agreement with that, we also observed that vaccinated mice that remained tumor-free after the tumor challenge for more than a month, were partially or completely immune to a secondary tumor challenge (data not shown).

II.5. Therapy of established tumors with the hgp100-GM-CSF DNA vaccine.

The experiments described above showed that gp100 gene vaccination of naive mice, especially in combination with GM-CSF, can result in induction of a T cell-mediated immune response which is capable of protecting mice from a subsequent tumor challenge. To approximate the experimental conditions to clinical situation, we investigated the therapeutic effect of the GM-CSF-gp100 gene therapy in mice bearing established tumors. Mice were injected i.d. in the middle of the abdomen with B16-gp100 tumor cells. Seven days later, when the tumors reached about 4 mm in diameter, mice received a combined GM-CSF-gp100 treatment at two sides of abdomen. This treatment was repeated on day 10, 13 and 17 post tumor cell implantation. The results in Fig.10A show that the GM-CSF-gp100 gene combination induced suppression of tumor growth when compared to control mice ($P < 0.05$ starting from day 17 of tumor growth).

The effect of gp100-GM-CSF gene therapy against established tumors was further confirmed by the extended survival of mice (Fig.10B). The survival was calculated based on the days the mice were sacrificed when their tumor diameter reached 15 mm. Thus, untreated tumor-bearing mice and mice treated with the empty vector + GM-CSF cDNA survived for 26.11 ± 0.96 and 25.5 ± 1.42 days, respectively, whereas gp100 + GM-CSF cDNA-treated mice survived for 37.5 ± 2.72 days ($P < 0.005$). The experiment depicted in Fig.5 was repeated two times, with the treatment started on day 4 or on day 7 post tumor cell implantation, and similar results were obtained.

II.6. RT-PCR analysis of recurrent tumors in vaccinated mice.

Although gp100 gene vaccination, especially in combination with GM-CSF, resulted in tumor protection, tumors relapsed in some mice after several weeks post tumor challenge. It could be that B16-gp100 tumor cells injected into vaccinated mice have lost expression of hgp100 and, therefore, became resistant to hgp100-specific T cell immune response. To address this question, we analyzed the expression of hgp100 RNA in tumors that developed in vaccinated mice at different times after the first or second tumor challenge. The RT-PCR analysis results presented in Fig.11 show that tumors which developed in vaccinated mice shortly after the challenge, 4 weeks after the challenge, or even 4 weeks after the second challenge, continued to express hgp100 RNA.

III. Attempt to Develop Dendritic Cell Gene Transfer System

III.1. Dendritic cell cultures

The system for dendritic cell (DC) culture and characterization has been established. We are able

to obtain about 30 million DCs from each sample of 100 million peripheral blood mononuclear cells (PBMC) over a 5 to 7-day culture period. A flow cytometry analysis system (allowing analysis of 9 markers at the same time) has been used to efficiently characterize these PBMC-derived DCs. The DCs generated in our culture system were over 50% pure based on morphology and the expression of a profile of CD3-, CD14-, CD16-, CD19-, CD20-, CD56-, HLA-DR+, and CD123+ or CD11c+ as assessed by flow cytometry.

In order to reduce the background in the experiment using DCs as the antigen presenting cells (e.g. GP-100 induced CTL assay), we have tried different serum supplement in DC culture media instead of fetal bovine serum (FBS). The results show that the donor's own plasma provides a good support of DC growth, even though the percentage of mature DC is lower than that in the culture with FBS.

III.2. Gene transfer into DCs

Our previous attempts are unsuccessful in using transiently GP-100 transfected DCs as APC to elicit CTL against melanoma cell lines and GP-100 expressing clones, partially because of the insufficient GP100 expression in DCs. Experiments are in progress to optimize conditions to transiently transfect DCs with cDNA for GP-100. We have evaluated Gene-Porter's technique (Lipofection method) and QIAGEN's technique (unknown chemical method) comparing to genegun. Currently, gene gun still shows the results superior to the other two techniques. Transgene expression level in DCs is from 1% to 7% by flow cytometry analysis. We also confirmed that the low end 1% expression is "real" by visualizing the expression of green fluorescence protein gene under microscope. We are working on improving our vector for GP-100 gene by replacing the previous vector with a new Gene Gripä vector, with a hope that the new construct will help to enhance the GP-100 expression on DCs.

Another approach is to enhance the antigen presenting potential of DCs through the modification of culture regimen. Lipopolysaccharide (LPS), Mycobacterium bovis bacillus Calmette-Guerin (BCG), or Diphtheria/Tetanus/Pertussis will be evaluated separately for their ability to induce DC maturation in culture. In addition, we have tested several herbal extracts for their effects on dendritic cell growth and function. We will observe DCs in their reduction of endocytosis, expression of CD83 and B7 costimulatory molecules and IL-12 production.

B3. Discussion

I. Combinational (IL-12, pro-IL-18, ICE) Gene Therapy Approach

It is also noteworthy that we show that the triple gene set of IL-12, pro-IL-18 and ICE in combination can be successfully delivered in vitro and in vivo by particle-mediated gene transfer with a gene gun device. In addition, in terms of induction of IFN- γ the finding that transfection of pro-IL-18 and ICE cDNA is superior to that of pro-IL-18 cDNA alone suggests that the two testing genes were concomitantly expressed in the same cell, because cleavage of pro-IL-18 by ICE requires the expression of both transgenes within the same cell (15,16). Thus, particle-mediated gene transfer effectively results in the expression of at least two or perhaps three transgenes in the same cells both in vitro and in vivo. Theoretically, this technique enables multiple gene transfer and expression in the same cells by co-precipitation of multiple species of DNA molecules onto the same gold beads (18,19).

Previous reports demonstrated efficacy of the combination of three testing genes in a vaccination

model (20). The combination of IL-18 and IL-12 protein therapy in a murine model has also been reported. Although tumor regression was noted, the treatment related toxicity was very high (21). In contrast, no adverse effect was observed in treated mice in our experiments using the combination gene therapy protocol with a gene gun. This suggests that gene gun-mediated local delivery of IL-12 / pro-IL-18 / ICE genes may be clinically desirable and safe as a strategy for local cancer therapy.

Results from our present study also demonstrate that IL-12 gene therapy can induce substantially more potent antitumor effects on the established tumor than the IL-18 gene therapy. In TS/A tumor-bearing mice receiving gene gun treatment of IL-12 cDNA, 17.4 % of mice showed a complete regression of the established tumors. In contrast, IL-18 gene therapy as administered here has failed to eradicate tumors completely and all IL-18 cDNA treated mice eventually died from progression of testing tumors (Table 1). Others have also reported that the antitumor effects of SCK cells (murine mammary adenocarcinoma) expressing IL-12 were more striking than those of SCK cells expressing IL-18 in tumor protection model (20). Importantly, we also showed in this study that the potent antitumor effects induced by IL-12 / pro-IL-18 / ICE cDNA were in fact reflected by the prolongation of survival time in treated animals. When mice whose tumor did not exceed 100 mm² in size were defined as survivors, in the groups of mice receiving gene therapy with IL-12 / pro-IL-18 / ICE, pro-IL-18 / IL-12, and IL-12 alone, 59.1%, 30.4% and 17.4% of mice were found to survive on day 50, respectively.

Our finding that the combinational gene therapy requires mainly CD8+ T cells is similar to the results of antibody depletion experiments for animals receiving IL-12 protein or gene therapy alone (2,6). Micallef et al. demonstrated that the effector cells responsible for antitumor effects of pretreatment with rIL-18 were NK cells in the initial phase, both CD4+ and CD8+ T cells in the second phase (between day 9 and 5), and CD4+ T cells for the long term immunological memory (22, 23). Our results indicate that the combination therapy of IL-12 and IL-18 can cause antitumor effects primarily via activation of CD8+ T cells.

Result of IFN- γ induction experiments suggests that IFN- γ is, in part, responsible for the antitumor efficacy of the combination gene therapy of IL-12 / pro-IL-18 / ICE. The lack of complete abrogation of antitumor efficacy in animals treated with anti-IFN- γ Ab may be explained by persistent local IFN- γ even in the mice treated with anti-IFN- γ Ab. Moreover, it is likely that IFN- γ may be a important, but not necessarily essential intermediate in this antitumor cascade. This is supported by studies that show that the use of the IFN- γ gene in similar tumor treatment models has not resulted in tumor regression (7), implying that IL-12 and this combination gene therapy can have immunologically mediated antitumor effects other than IFN- γ release.

In summary, gene gun-mediated transfer of expression vectors for IL-12, pro-IL-18 and ICE cDNA can confer a synergistic induction of IFN- γ in vitro and in vivo. Combination of these three genes can effectively result in complete regression of TS/A tumors, which effect is superior to either IL-12 or pro-IL-18 cDNA alone. This antitumor response can be completely blocked by CD8+ T cell depletion, and partially abrogated by antibodies to IFN- γ . In addition, this combination gene therapy can induce tumor-specific immunological memory. To our knowledge, this is the first report on the successful use of a triple gene combination for cancer therapy using non-viral vectors. These findings suggest that combination gene therapy of IL-12, IL-18 and ICE cDNA may provide a potential application for cancer gene immunotherapy, and the current gene gun delivery approach may provide a new methodology for effective and functional delivery of multiple, candidate therapeutic genes for experimental and potential clinical applications.

II. Gp100+GM-CSF Gene Vaccination Approach

Genetic vaccination against cancer seems to have a great potential as a simple and yet effective way of inducing a protective immune response. We show in this study that gene gun-mediated vaccination of mice with hgp100 plasmid cDNA, especially in combination with mGM-CSF cDNA, resulted in effective protection against hgp100-expressing melanoma. Importantly, as low as 62.5 ng of hgp100 plasmid was sufficient to achieve a substantial level of tumor protection when combined with the mGM-CSF plasmid.

Immunostimulatory peptides of hgp100 have been recently synthesized and modified to become more immunogenic (17, 24). These peptides were found to induce a protective immune response in mice (24) and in cancer patients (25,26). Remarkably, an initial study has indicated that 42% of melanoma patients demonstrated objective responses as a result of gp100 peptide vaccination followed by a course of treatment with recombinant IL-2 (27). Thus, vaccination against the gp100 antigen seems to be an effective approach for melanoma treatment. Another way to achieve an immune response against gp100 is genetic immunization with gp100 cDNA. DNA immunization could potentially be more effective than peptide immunization, as has been recently indicated in a comparative experimental study using a model tumor antigen (28). Genetic vaccination with naked hgp100 plasmid DNA resulted in generation of CTL and protection against hgp100-expressing B16 tumors (29). Our results confirm these findings and demonstrate that in order to achieve comparable antitumor effect, gene gun-mediated gene delivery required at least 20 times less hgp100 DNA (2.5 µg) than intramuscular injection of naked DNA (29).

Our data demonstrate, in addition, that the effective dose of gp100 DNA can be reduced 40 times by co-administration with the gene encoding for mGM-CSF. An adjuvant effect of GM-CSF DNA for experimental cancer vaccination has previously been reported (30). The mechanism of this adjuvant effect may be attributed to the ability of GM-CSF to induce and activate antigen-presenting cells, such as dendritic cells and macrophages, and thereby potentiate a specific immune response elicited by tumor vaccines. At least in some tumor systems, GM-CSF was found to be the most potent adjuvant as compared with other cytokines genes (31). In agreement with those findings, our preliminary results indicate that GM-CSF is more potent in enhancing the protective effect of gp100 DNA vaccination than IL-4 or B7.1 DNA (data not shown).

A theoretical disadvantage of a vaccination approach based on using a specific TAA peptide or plasmid DNA encoding a TAA is the possibility of tumor escape from the immune control by developing "antigen loss" or antigen-negative tumor variants. Indeed, in a clinical study using gp100 peptide vaccination, it was observed that a patient initially responded to the vaccination by tumor shrinkage but later the tumor relapsed (32). This relapsed tumor has lost the expression of gp100 and was resistant to gp100-specific CTL. Our data on experimental gp100 gene vaccination also provide the evidence that the tumors may escape from the T cell-mediated immune control. However, we found that the relapsing tumors, being insidious for several weeks in vaccinated mice, continued to express hgp100 RNA. Although this observation does not exclude the loss of gp100 on the protein level, it is highly unlikely that the tumor cells would develop a new mechanism to regulate a gene on a post-transcriptional level rather than by simply deleting it. It is possible, instead, that when the immune response to gp100 weakens below certain threshold, dormant tumor cells from a challenge inoculum begin to grow. Future experiments are warranted to determine the mechanism of tumor escape in gp100-vaccinated mice.

C. KEY RESEARCH ACCOMPLISHMENTS.

- Gene gun-mediated delivery of a triple gene combination, IL-12, pro-IL-18 and ICE, resulted in a synergistic immune activation *in vitro* and reduction of breast tumor growth *in vivo*
- Gene gun-mediated combinational approach for gene vaccination, using a tumor antigen cDNA (gp100) together with a cytokine cDNA (GM-CSF), was found to induce effective, T cell-mediated protection against tumors
- Small quantities (60 ng) of gp100 cDNA delivered by a gene gun were sufficient to induce tumor protection when combined with GM-CSF cDNA.

D. REPORTABLE OUTCOMES.

D.1. PRESENTATIONS AT THE MEETINGS

1. Rakhmilevich, A.L., K.Janssen and N-S. Yang. Gene gun-mediated IL-12 gene therapy for breast cancer in mice. *Cancer Gene Therapy*, 4:S52-S53, 1997. A poster presentation at 6th International Conference on Gene Therapy of Cancer, Nov 20-22, 1997, San Diego, CA.
2. Rakhmilevich, A.L., K.M.Wright, B.Javorsky, T. Roberts, M. R. Albertini, N-S. Yang, and P.M.Sondel. Pre-clinical In Vivo Gene Therapy Approaches Using Gene Gun. An oral presentation at 7th International Conference on Gene Therapy of Cancer, Nov 19-21, 1997, San Diego, CA.
3. N-S. Yang, A.L.Rakhmilevich, P.M.Sondel, D.Mahvi, G.Hogge, and G.MacEwen. Cytokine gene therapy for cancer and DNA cancer vaccines: A gene gun approach. An oral presentation at 1998 Keystone Symposium "Synthetic Non-Viral Gene Delivery Systems", January 19-25, 1998.
4. N-S. Yang, K. Oshikawa, D. Mahvi, F-S. Shi, M.Imboden, M.D.Macklin, M.R.Albertini, T.Roberts, K.M.Wright, P.M.Sondel, and A.L.Rakhmilevich. Gene gun-mediated DNA cancer vaccination with combinational cytokine gene therapy, and tumor-associated antigen and GM-CSF. 2nd Annual Meeting, American Society of Gene Therapy, June 9-13, 1999.

D.2. PUBLICATIONS

1. Rakhmilevich A.L., Timmins J.G., Janssen K., Pohlmann E.L., Sheehy M.J. and Yang N-S. Gene gun-mediated IL-12 gene therapy induces antitumor effects in the absence of toxicity: a direct comparison with systemic IL-12 protein therapy. *J. Immunother.*, 22, 135-144 (1999).
2. Rakhmilevich A.L., Janssen K., Hao, Z., Sondel P.M. and Yang N-S. Interleukin 12 gene therapy of a weakly immunogenic mouse mammary carcinoma results in reduction of spontaneous lung metastases via a T cell-independent mechanism. 1999 (in press).
3. Rakhmilevich A.L. and Yang N-S. In vivo particle-mediated gene transfer for cancer therapy. In *Gene Therapy of Cancer: Methods and Protocols*, editors Walther and Stein, Berlin (in press).
4. Oshikawa K., Shi F., Rakhmilevich A.L., Sondel P.M., Mahvi D.M., and Yang N-S.

Synergistic Inhibition of Tumor Growth in a Murine Mammary Adenocarcinoma Model by Combinational Gene Therapy Using Interleukin-12, pro-Interleukin-18 and IL-1 β -Converting Enzyme cDNA. *Proc. Natl. Acad. Sci. USA*, (in press.)

E. CONCLUSIONS

- A combinational delivery of IL-12, pro-IL-18 and ICE cDNA confers a synergistic effect on IFN γ and IL-18 production in vitro and in vivo.
- A combinational gene therapy with IL-12, pro-IL-18 and ICE cDNA confers a synergistic effect against TS/A breast tumor, resulting in extended survival of mice.
- Cutaneous vaccination of mice with a combination of hgp100 cDNA and mGM-CSF cDNA via gene gun results in a synergistic tumor protection.
- Tumor protection induced by gp100 + GM-CSF DNA vaccination depends on T cells.
- Skin delivery of gp100 + GM-CSF DNA results in suppression of growth of established mouse melanoma, followed by extended survival of treated mice.

F. REFERENCES

1. Lippman, M. The Development of Biological Therapies for Breast Cancer. *Science* 259, 631-632 (1993).
2. Brunda, M., Luistro, L., Warriar, R., Wright, R., Hubbard, B., Murphy, M., Wolf, S. and Gately, M. Antitumor and Antimetastatic Activity of Interleukin 12 against Murine Tumors. *J Exp. Med.* 178, 1223-1230 (1993).
3. Nastala, C., Edington, H., McKinney, T., Tahara, H., Nalesnik, M., Brunda, M., Gately, M., Wolf, S., Schreiber, R., Storkus, W. and Lotze, M. Recombinant IL-12 Administration Induces Tumor Regression in Association with IFN- γ Production. *J Immunology* 153, 1697-1706 (1994).
4. Orange, J., Salazar-Mather, T., Opal, S., Spencer, R., Miller, A., McEwen, B. and Biron, C. Mechanism of Interleukin 12-mediated Toxicities during Experimental Viral Infections: Role of Tumor Necrosis Factor and Glucocorticoids. *J. Exp. Med.* 181, 901-914 (1995).
5. Marshall, E. Cancer Trial of Interleukin-12 Halted. *Science* 268, 1555 (1995).
6. Rakhmievich, A.L. *et al.* Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors. *Proc. Natl. Acad. Sci. USA* 93, 6291-6296 (1996).
7. Rakhmievich, A.L., Janssen, K., Turner, J., Culp, J. & Yang, N.-S. Cytokine gene therapy of cancer using gene gun technology: Superior antitumor activity of IL-12. *Human Gene Therapy* 8, 1303-1311 (1997).

8. Rakhmilevich A.L., Timmins J.G., Janssen K., Pohlmann E.L., Sheehy M.J. and Yang N-S. Gene gun-mediated IL-12 gene therapy induces antitumor effects in the absence of toxicity: a direct comparison with systemic IL-12 protein therapy. *J. Immunother.*, 22, 135-144 (1999)
9. Nanni, P., De Giovanni, C., Lollini, P.-L., Nicoletti, G. and Prodi, G. TS/A: a New Metastasizing Cell Line from a BALB/c Spontaneous Mammary Adenocarcinoma. *Clin. Exp. Metast.* 1, 373-380 (1983).
10. Cavallo, F., Di Pierro, F., Giovarelli, M., Gulino, A., Vacca, A., Stoppacciaro, A., Forni, M., Modesti, A. and Forni, G. Protective and Curative Potential of Vaccination with Interleukin-2 Gene-transfected Cells from a Spontaneous Mouse Mammary Adenocarcinoma. *Cancer Res.* 53, 5067-5070 (1993).
11. Aslakson, C. and Miller, F. Selective Events in the Metastatic Process Defined by Analysis of the Sequential Dissemination of Subpopulations of a Mouse Mammary Tumor. *Cancer Res.* 52, 1399-1405 (1992).
12. Hossain, A., Sarkar, A. and Sarkar, N. Mixed Inocula of Mouse Mammary Tumour Cell Subpopulations Result in Changes of Organ-Specific Metastasis. *Clin. Expl. Met.* 9, 6, 501-515 (1991).
13. Lollini, P. L., Bosco, M., Cavallo, F., De Giovanni, C., Giovarelli, M., Landuzzi, L., Musiani, P., Modesti, A., Nicoletti, G., Palmieri, G., Santoni, A., Young, H., Forni, G. and Nanni, P. Inhibition of Tumor Growth and Enhancement of Metastasis after Transfection of the g-Interferon Gene. *Int. J. Cancer* 55, 320-329 (1993).
14. Okamura, H., Tsutsui, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukata, Y., Hattori, K. & et. al. (1995) *Nature*. **378**, 88-91.
15. Ghayur, T., Banerjee, S., Hugunin, M., Butler, D., Herzog, L., Carter, A., Quintal, L., Sekut, L., Talanian, R., Paskind, M. & et. al. (1997) *Nature*. **386**, 619-623.
16. Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Hsiao, K., Fleming, M, A., Hayashi, N., Higashino, K., Okamura, H., Nakanishi, K. & et. al. (1997) *Science*. **275**, 206-209.
17. Schreurs, M.W., de Boer, A.J., Schmidt, A., Figdor, C.G. and Adema, G.J. Cloning, expression and tissue distribution of the murine homologue of the melanocyte lineage-specific antigen gp100. *Melanoma Research*, 7, 463-470 (1997).
18. Sun, W. H., Burkholder, J. K., Sun, J., Culp, J., Turner, J., Lu, X. G., Pugh, T. D., Ershler, W.B. & Yang, N. S. (1995) *Proc. Natl. Acad. Sci. USA*. **92**, 2889-2893.
19. Albertini, M. R., Elmer, C. A., Schell, K., Tans, K. J., King, D. M. & Sheehy, M. J. (1996) *Cancer Gene Therapy*. **3**, 192-201.
20. Coughlin, C. M., Salhany, K. E., Wysocka, M., Aruga, E., Kurzawa, H., Chang, A. E., Hunter, C. A., Fox, J. C., Trinchieri, G. & Lee, W. M. F. (1998) *J. Clin. Invest.* **101**, 1441-1452.
21. Osaki, T., Peron, J. M., Cai, Q., Okamura, H., Robbins, P. D., Kurimoto, M., Lotze, M. T. & Tahara, H. (1998) *J. Immunol.* **160**, 1742-1749.

22. Micallef, M. J., Tanimoto, T., Kohno, K., Ikeda, M., and Kurimoto, M. (1997) *Cancer Res.* **57**, 4557-4563.
23. Micallef, M. J., Yoshida, K., Kawai, S., Hanaya, T., Kohno, K., Arai, S., Tanimoto, T., Torigoe, K., Fujii, M., Ikeda, M. & et. al. (1997) *Cancer Immunol. Immunother.* **43**, 361-367.
24. Salgaller, M.L., Marincola, F.M., Cormier, J.N., and Rosenberg, S.A. Immunization against epitopes in the human melanoma antigen gp100 following patient immunization with synthetic peptides. *Cancer Res.*, **56**, 4749-4757 (1996).
25. Bakker, A.B.H., Schreurs, W.J., de Boer, A.J., Kawakami, Y., Rosenberg, S.A., Adema, G.J., and Figdor, C.G. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J. Exp. Med.*, **179**, 1005-1009 (1994).
26. Kawakami, Y., Eliyahu, S., Jennings, C., Sakaguchi, K., Kang, X., Southwood, S., Robbins, P.F., Sette, A., Appella, E. and Rosenberg, S.A. Recognition of multiple epitops in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J. Immunol.*, **154**, 3961-3968 (1995).
27. Rosenberg, S.A., Yang, J.C., Schwartzentruber, D.J., Hwu, P., Marincola, F.M., Topalian, S.L., Restifo, N.P., Dudley, M.E., Schwarz, S.L., Spiess, P.J., Wunderlich, JR, Parkhurst, M.R., Kawakami, Y., Seipp, C.A., Einhorn, J.H. and White, D.E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nature Medicine* **4**: 321-332 (1998).
28. Ross HM. Weber LW. Wang S. Piskun G. Dyal R. Song P. Takechi Y. Nikolic-Zugic J. Houghton AN. Lewis JJ. Priming for T-cell-mediated rejection of established tumors by cutaneous DNA immunization. *Clinical Cancer Research*. 3(12 Pt 1):2191-6, 1997.
29. Schreurs MW. de Boer AJ. Figdor CG. Adema GJ. Genetic vaccination against the melanocyte lineage-specific antigen gp100 induces cytotoxic T lymphocyte-mediated tumor protection. *Cancer Research*. 58(12):2509-14, 1998.
30. Conry RM. Widera G. LoBuglio AF. Fuller JT. Moore SE. Barlow DL. Turner J. Yang NS. Curiel DT. Selected strategies to augment polynucleotide immunization. *Gene Therapy*. 3(1):67-74, 1996.
31. Dranoff G. Jaffee E. Lazenby A. Golumbek P. Levitsky H. Brose K. Jackson V. Hamada H. Pardoll D. Mulligan RC. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA*. 90(8):3539-43, 1993
32. Lee KH. Panelli MC. Kim CJ. Riker AI. Bettinotti MP. Roden MM. Fetsch P. Abati A. Rosenberg SA. Marincola FM. Functional dissociation between local and systemic immune response during anti-melanoma peptide vaccination. *Journal of Immunology*. 161(8):4183-94, 1998.

FIGURE LEGENDS

Figure 1. IFN- γ secretion in splenocytes in vitro induced by cytokine proteins secreted from

transfected COS-7 (A) and TS/A (B) cells. IL-18 bioassay (stimulation of IFN- γ release) was carried out as described in Materials and Methods. Means \pm S.D. were calculated from triplicates. *, $P < 0.05$ versus pro-IL-18 alone in both cells; †, $P < 0.0001$ versus the other groups in COS-7 cells; ‡, $P < 0.005$ versus the other groups in TS/A cells. A, IL-12 cDNA; B, pro-IL-18 cDNA; C, pro-IL-18 / ICE cDNA; D, IL-12 / pro-IL-18 cDNA; E, IL-12 / pro-IL-18 / ICE cDNA; F, control plasmid (pNGVL-3).

Figure 2. IFN- γ production in the treated skin tissue (A) and from splenocytes (B) after gene gun treatment. A) Skin overlying TS/A tumor was excised after the second gene gun treatment and IFN- γ in the skin tissue lysate was measured by ELISA. *, $P < 0.05$ versus all other treatment groups. Mean \pm S.D. are shown for 4 mice per group. B) Splenocytes (2×10^6) were isolated after the second gene gun treatment and cultured in 2 ml of RPMI 1640 for 24 hours, †, $P < 0.05$ versus pro-IL-18 alone; ‡, $P < 0.05$ versus all other groups. Mean \pm S.D. are shown for 4 mice per group. A, IL-12 cDNA; B, pro-IL-18 cDNA; C, pro-IL-18 / ICE cDNA; D, IL-12 / pro-IL-18 cDNA; E, IL-12 / pro-IL-18 / ICE cDNA; F, control plasmid (pNGVL-3).

Figure 3. TS/A growth following *in vivo* gene gun treatment. The protocol of gene gun treatment was described in Materials and Methods. Arrows indicate the days when gene gun treatments were performed. Mean tumor size \pm SEM are shown for 15 mice per each group. A statistically significant difference in suppression of the tumor growth was observed in the group treated with IL-12 / pro-IL-18 / ICE, compared with that from pro-IL-18 alone ($p < 0.05$ on day 12, and $p < 0.0001$ on day 14 to 24), from pro-IL-18 / ICE ($p < 0.001$ on day 14 and $p < 0.0001$ on day 16 to 24), from IL-12 alone ($p < 0.05$ on day 22 and $p < 0.01$ on day 24), and from the control plasmid ($p < 0.0001$, day 12 to 24). □, IL-12 cDNA; •, pro-IL-18 cDNA; Δ, pro-IL-18 / ICE cDNA; ∇, IL-12 / pro-IL-18 cDNA; ●, IL-12 / pro-IL-18 / ICE cDNA; ○, control plasmid (pNGVL-3).

Figure 4. Effects of *in vivo* depletion of CD4⁺ and CD8⁺ subsets of T cells and neutralization of IFN- γ on the antitumor response induced by combinatorial gene therapy with IL-12, pro-IL-18 and ICE. A) Mixture of anti-CD4 and anti-CD8 mAb, or anti-IFN- γ mAb were injected intraperitoneally. Mean tumor size \pm SEM are shown for 5 mice per group. ∇, anti-CD4 and anti-CD8 mAb; Δ, anti-IFN- γ mAb; ●, no mAb; □, rat IgG. All above groups (∇, Δ, ● and □) received IL-12 / pro-IL-18 / ICE cDNA treatment. ○, control plasmid (pNGVL-3) and no mAb. B) Anti-CD4 and anti-CD8 mAb were administered separately. Mean tumor size \pm SEM are shown for 6 mice per group. ∇, anti-CD4 mAb; Δ, anti-CD8 mAb; ●, no mAb; □, rat IgG. All above groups (∇, Δ, ● and □) received IL-12 / pro-IL-18 / ICE cDNA treatment. ○, control plasmid (pNGVL-3) and no mAb.

Figure 5. Induction of CTL activities in mice with complete regression was observed following gene therapy with IL-12, IL-12 / pro-IL-18, or IL-12 / pro-IL-18 / ICE. The representative data of two independent experiments are shown. □, IL-12 cDNA; Δ, IL-12 / pro-IL-18 cDNA; ●, IL-12 / pro-IL-18 / ICE cDNA; ○, control plasmid (pNGVL-3).

Figure 6. Expression of hgp100 in B16-gp100 melanoma cells and in the skin following vaccination with hgp100 plasmid cDNA. A. Mouse B16 melanoma cells (B16-wild), or the cell clone derived from B16 cells following gene gun-mediated transfection with human gp100 cDNA and selection in G418-containing media (B16-gp100), were used for flow cytometry. The cells were first permeabilized, then incubated with HMB-45 mAb reactive with human gp100, and

stained with FITC-conjugated goat anti-mouse IgG. *B.* RT-PCR detection of human gp100 transcripts in the skin after gene gun transfection with hgp100 cDNA. The abdominal skin area of C57BL/6 mice was transfected into with 625 ng of hgp100 plasmid DNA. Twenty four hr post transfection, skin samples were removed, RNA extracted and RT-PCR performed using the primers specific for the hgp100 sequence. *a*, Lane 1, hgp100 transcripts in control B16wt cell line, Lane 2, hgp100 transcripts in B16-gp100 stably transfected cell line, Lane 3, hgp100 transcripts in the skin from a control mouse transfected with empty vector, Lane 4, hgp100 transcripts in hgp100 -transfected skin, Lane 5, hgp100 transcripts in hgp100+GM-CSF transfected skin. *b*, internal control amplification using primers for the house keeping gene G3PDH in all the samples (Lane 1-5).

Figure 7. Gp100 gene vaccination: Protection against B16 melanoma. Skin of C57BL/6 mice was transfected at four sites with hgp100 cDNA (2.5 μ g/mouse), or hgp100 cDNA in combination with GM-CSF cDNA (2.5 μ g/mouse of each) using gene gun. Seven days later, vaccinated and naive mice were challenged i.d. with 5×10^4 parental B16 cells (B16-wild), or with 5×10^4 cells of the B16 cell clone stably expressing hgp100 (B16-gp100). Tumor growth was followed. Data are presented for 5 mice per group. Mice which did not develop tumors remained tumor-free for at least 60 days.

Figure 8. Dose effect of gp100 DNA, alone or in combination with GM-CSF DNA, on tumor protection. Skin of C57BL/6 mice was transfected using the gene gun with hgp100 cDNA (625, 62.5, or 6.25 ng/transfection/mouse) either alone or in combination with GM-CSF cDNA (625 ng). Control mice were transfected with the DNA encoding the empty vector, alone or in combination with GM-CSF DNA. Seven days later, vaccinated and naive mice were challenged intradermally with 5×10^4 cells of the B16 cell clone stably expressing hgp100 (B16-gp100). Tumor growth was followed. Data are presented for 5 mice per group, where each line represents one animal.

Figure 9. The role of T cells in tumor protection following gp100 + GM-CSF DNA vaccination. Skin of C57BL/6 mice was gene gun-transfected with hgp100 cDNA in combination with GM-CSF cDNA, 0.6 μ g/mouse of each (vaccine). A mixture containing 300 μ g of each anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) was administered intraperitoneally on days 6 and 11 after vaccination. Control groups received rat IgG (Sigma) at the dose 600 μ g/mouse per day. On day 7 post vaccination, vaccinated and naive mice were challenged i.d. with 5×10^4 cells of the B16 cell clone stably expressing hgp100 (B16-gp100). Tumor growth was followed. Data are presented for 5 mice per group.

Figure 10. Suppression of the growth of established tumors by the gp100+GM-CSF DNA vaccine. C57BL/6 mice were injected intradermally with 5×10^4 of B16-gp100 cells. On days 7, 10, 13 and 17 post-tumor cell implantation, the skin was transfected at two sites of the abdominal area using the gene gun with hgp100 cDNA in combination with GM-CSF cDNA, 625 ng of each plasmid per transfection site. Control mice received empty vector DNA + GM-CSF DNA. Kinetics of tumor growth was assessed by measuring the tumor diameter (A). The survival of mice was determined by the day the tumor diameter reached 15 mm (B). Each group consisted of 5 mice.

Figure 11. Detection of hgp100 transcripts in recurring tumors after vaccination with gp100 cDNA. Mice were vaccinated in the skin using gene gun with hgp100 cDNA alone or in combination with GM-CSF cDNA. Seven days later, the mice were challenged i.d. with 5×10^4 B16-gp100 cells. In one experiment, the vaccinated mice that remained tumor-free for 35 days

post tumor challenge, were challenged a second time with the same tumor. The intradermal tumors which developed in some vaccinated mice were later removed and analyzed by RT-PCR for expression of hgp100 RNA using hgp100-specific primers. *a*, Lane 1, hgp100 transcripts in the tumor which developed on day 8 after the first tumor challenge and was extracted on day 37. Lane 2, hgp100 transcripts in the tumor which developed on day 26 after first tumor challenge and was extracted on day 37. Lane 3, hgp100 transcripts in the tumor which developed on day 36 after secondary tumor challenge and was removed on day 43. Lane 4, hgp100 transcripts in B16-wild cells, Lane 5, positive control, hgp100 transcripts in B16-gp100 cell line. *b*, internal control amplification of house keeping gene G3PDH in all the samples (Lane 1-5).

Table 1. Complete regression of TS/A tumor

following gene gun treatment *.

Treatment	Mice with complete regression [†] / total	(%)
IL-12	4 / 23	17.4 [‡]
pro-IL-18	0 / 15	0 [¶]
pro-IL-18 / ICE	0 / 15	0 [¶]
IL-12 / pro-IL-18	6 / 23	26.1 [§]
IL-12/pro-IL-18/ ICE	11 / 22	50.0
Control (pNGVL-3)	0 / 23	0 [¶]

*Treatment protocol was same as that of Figure 5 (in IL-12, IL-12 / pro-IL-18, IL-12 / pro-IL-18 / ICE and control, 7 to 8 additional mice were evaluated). [†]The number of mice in which complete regression of established TS/A tumor was observed. [‡], P < 0.05 versus IL-12 / pro-IL-18 / ICE; [§], P = 0.089 versus IL-12 / pro-IL-18 / ICE; [¶], P < 0.0001 versus IL-12 / pro-IL-18 / ICE.

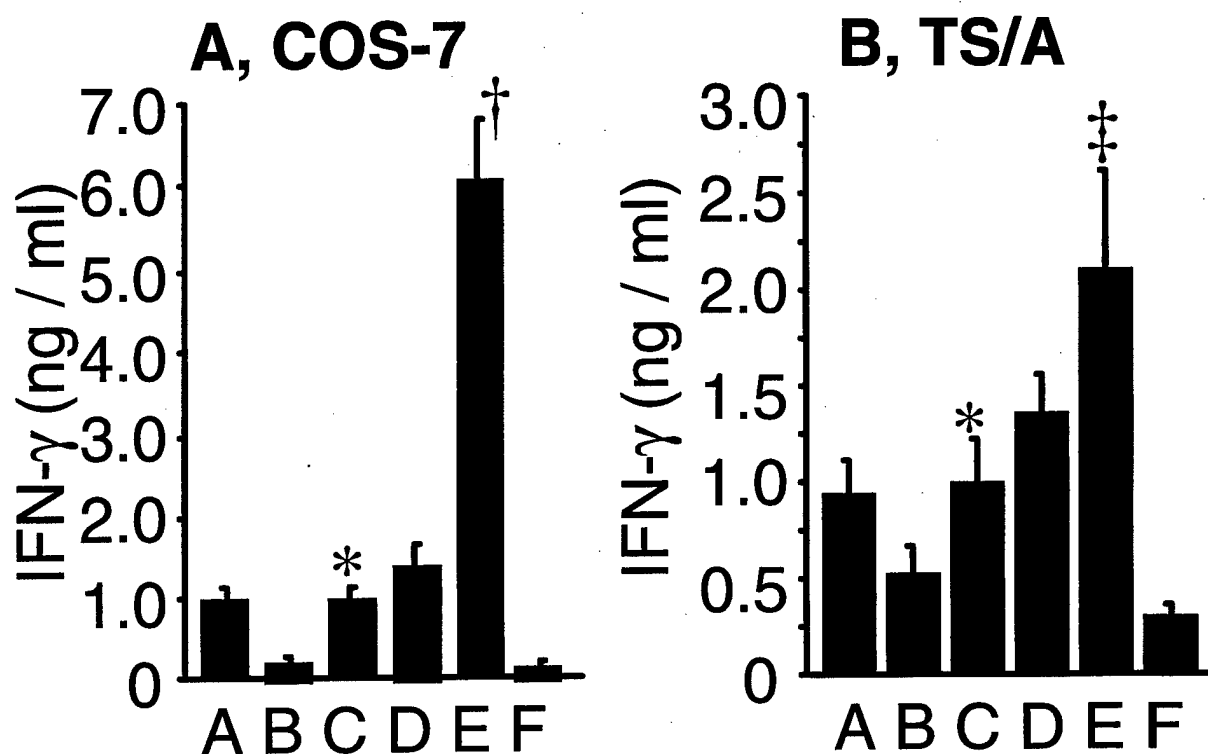


Fig. 1. IFN- γ production in splenocytes *in vitro* induced by cytokine proteins secreted from transfected COS-7 (A) and TS/A (B) cells.

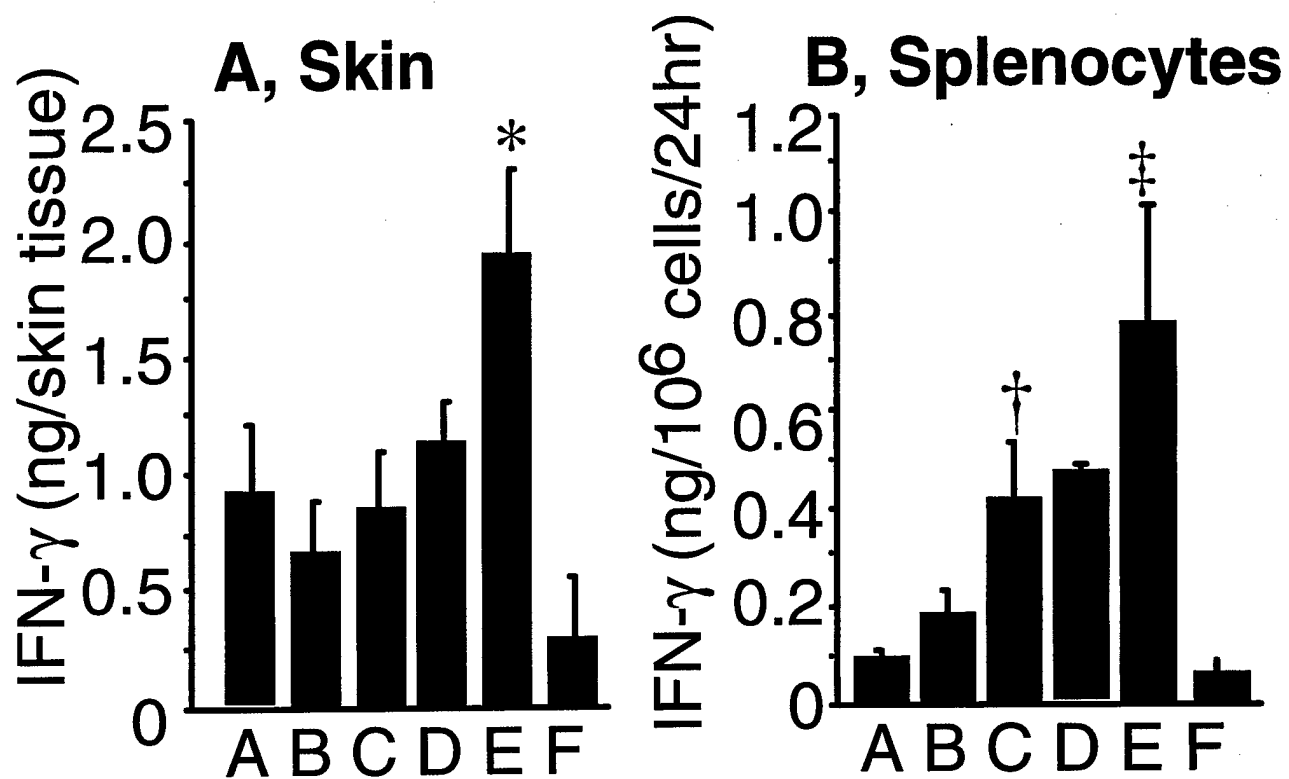


Fig. 2. IFN- γ production in the treated skin tissue (A) and from splenocytes (B) after gene gun treatment.

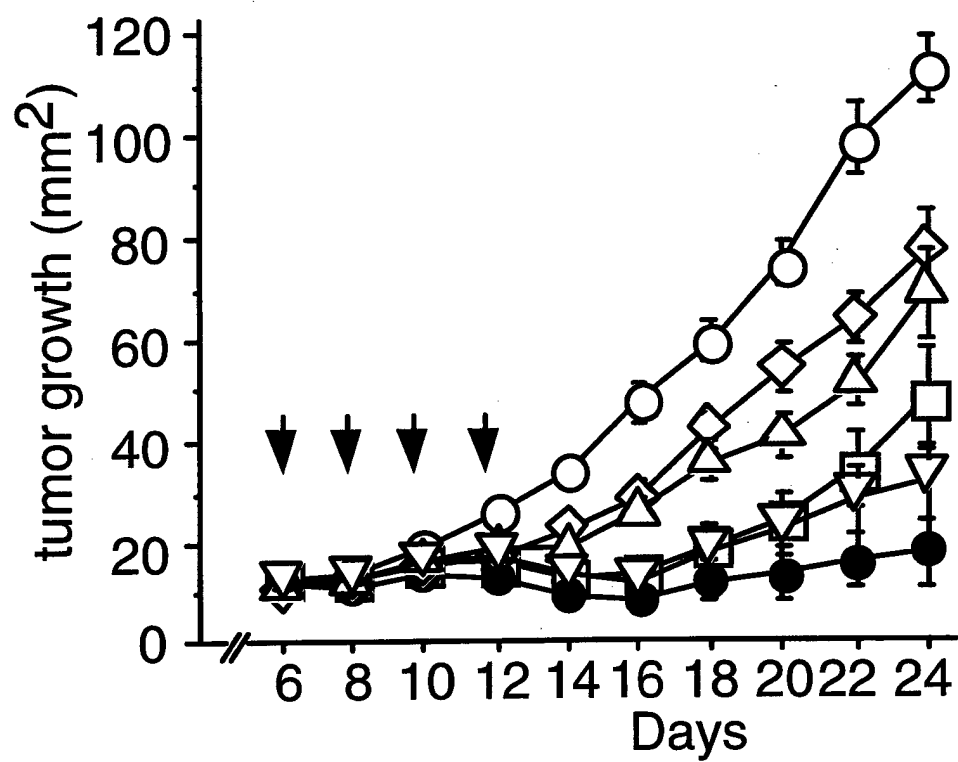


Fig. 3. TS/A growth following *in vivo* gene gun

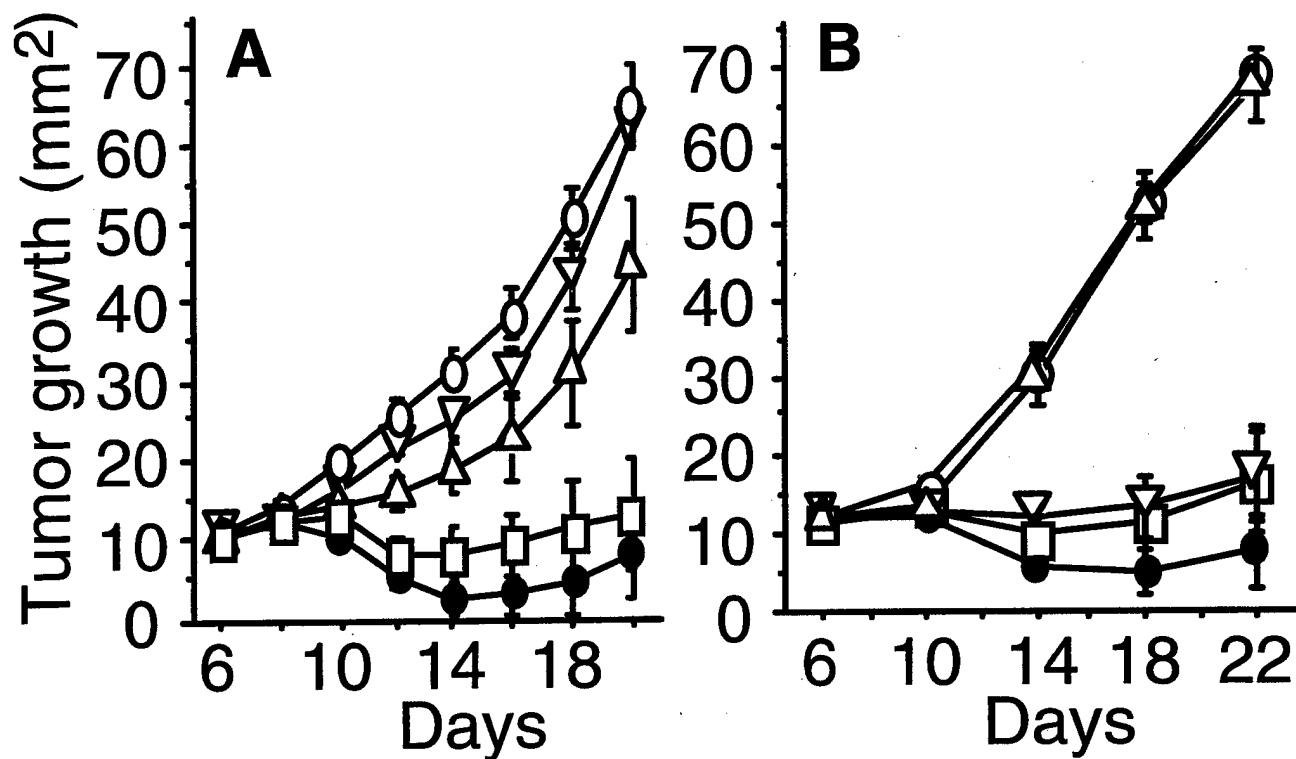


Fig. 4. Effects of *in vivo* depletion of CD4+ and CD8+ subsets of T cells and neutralization of IFN- γ on the antitumor response induced by combinatorial gene therapy with IL-12, pro-IL-18 and ICE.

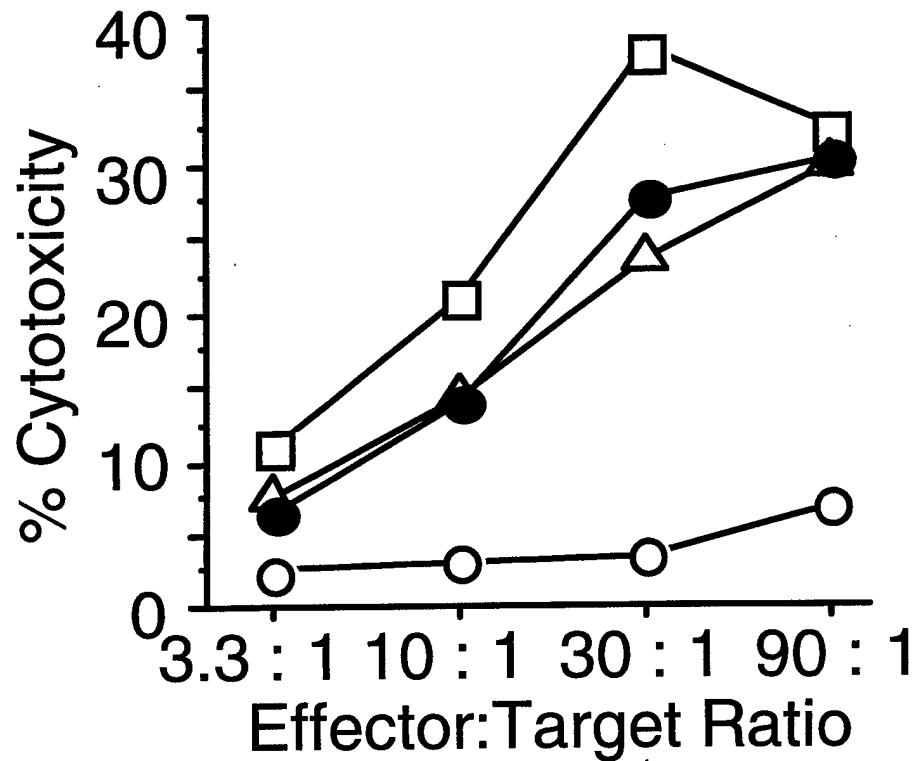
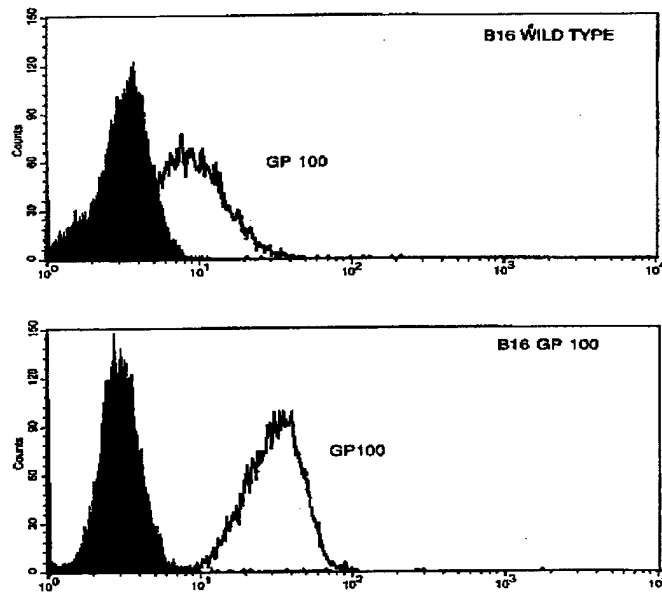


Fig. 5. Induction of CTL activities in mice with complete regression was observed following gene therapy with IL-12, IL-12 / pro-IL-18, or IL-12 / pro-IL-18 / ICE.

A



B



Fig.6. Expression of hugp100 in vitro

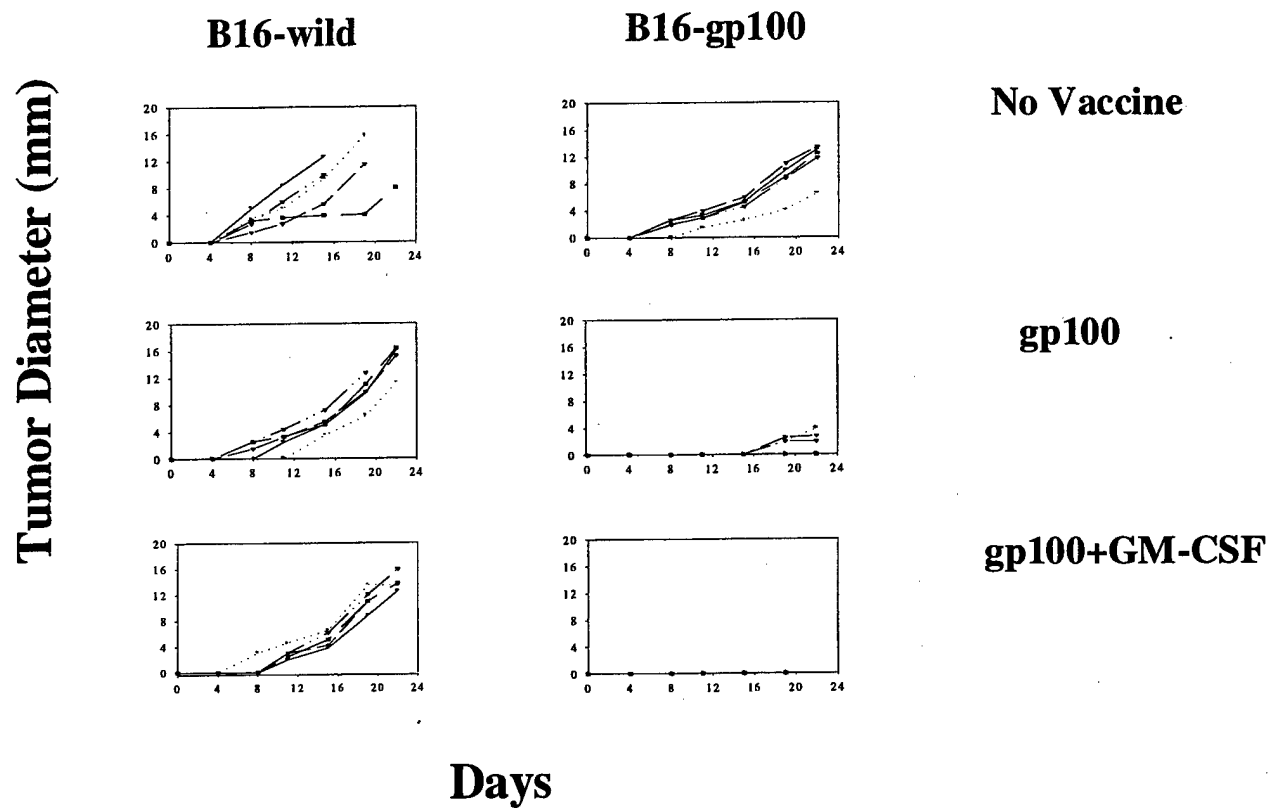


Fig. 7. Tumor protection following hugp100 DNA vaccination with or without mGM-CSF DNA vaccination in mice

Tumor Diameter (mm)

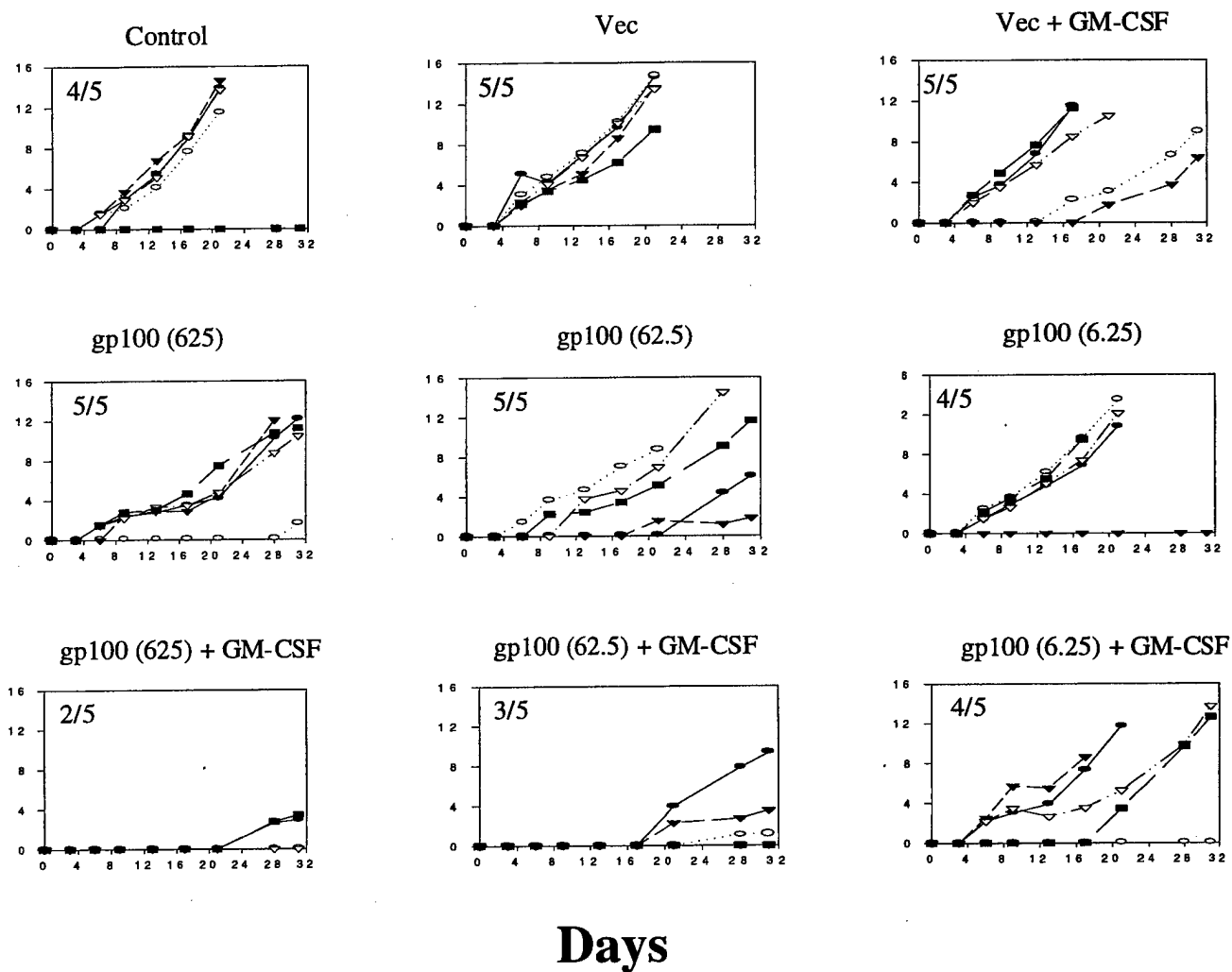


Fig.8. Dose effect of gp100 DNA vaccination

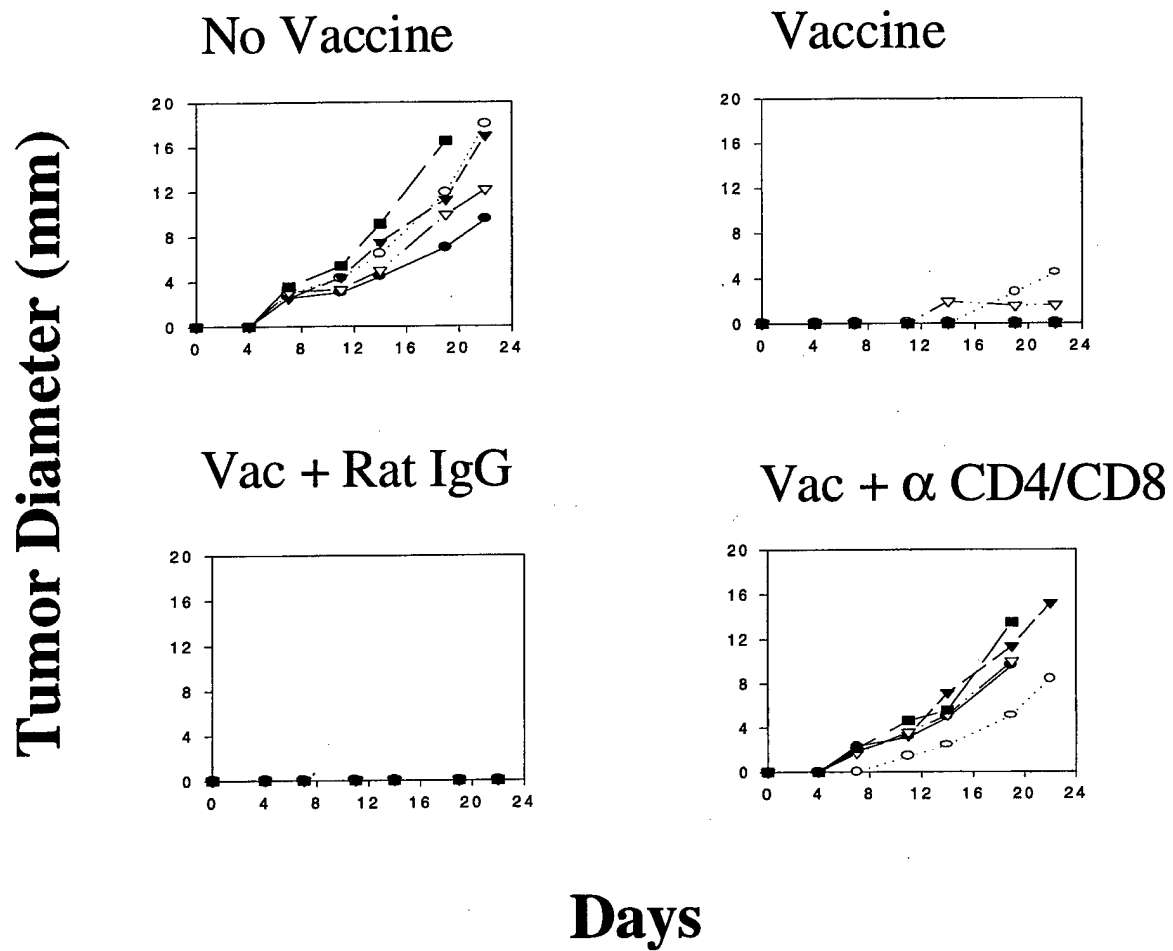


Fig. 9. Role of T cells in tumor protection induced by gp100+GM-CSF DNA vaccine

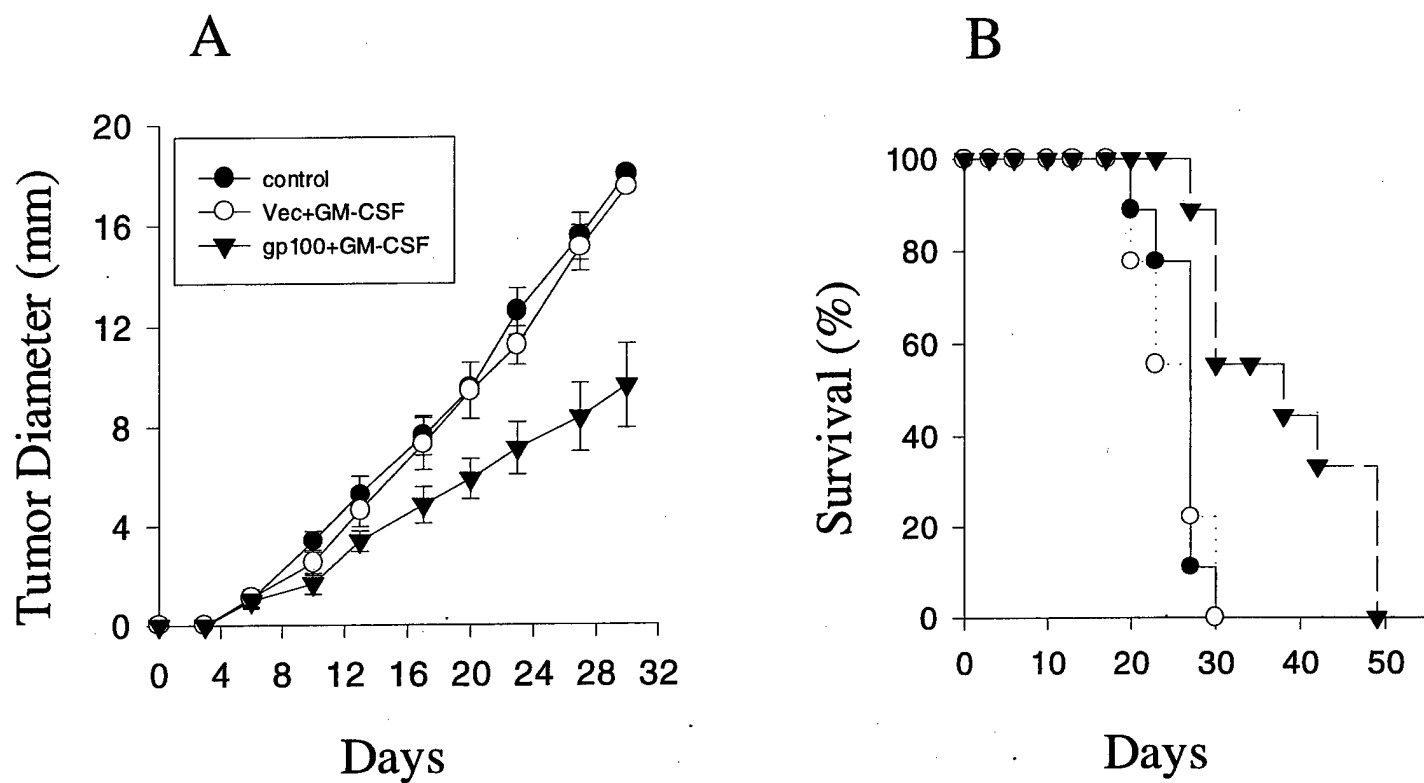


Fig. 10. Effect of gp100+GM-CSF gene vaccination against established tumors.

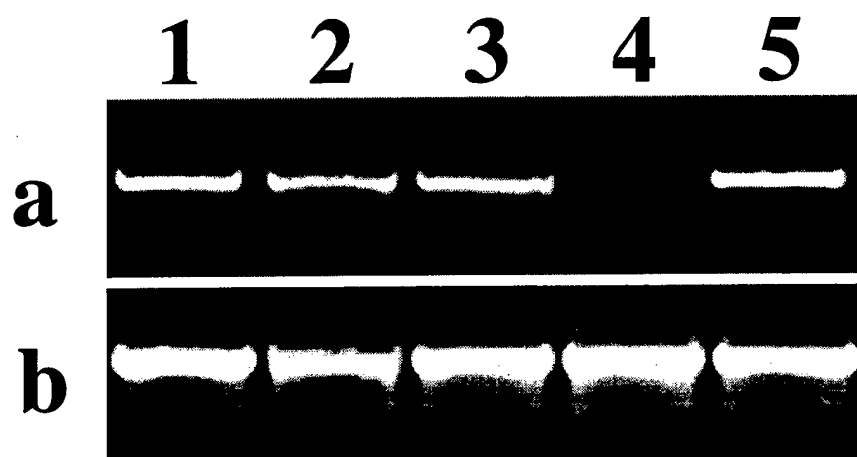


Fig. 11. Expression of hugp100 mRNA in tumors